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Design and Synthesis of Small Chemical Inhibitors Containing Different Scaffolds for *lck* SH2 Domain

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Abstract—On the basis of the structure of (*R*)-rosmarinic acid, a series of small chemical compounds with a different scaffold was synthesized as inhibitors for *lck* SH2 domain. From ELISA results, most of all chemical compounds showed a similar or a little lower binding activity for *lck* SH2 domain compared to the lead compound, (*R*)-rosmarinic acid. It was characterized that the backbone rigidity between two catechol substructures was required for the full activity and acid substructure of the lead compound was important for the activity. We successfully identified novel lead compounds that did not contain phosphotyrosine moiety and might have an improved bioavailability as inhibitor for *lck* SH2 domain.

Many of intracellular signaling pathways are mediated by phosphorylation and dephosphorylation of tyrosine residue of intracellular protein, controlled by kinase and phosphatase.[1] SH2 domains in kinase and phosphatase play pivotal roles in organizing coherent signal transducing complexes that are essential for the appropriate cellular response to extracellular stimuli.[1] Thus, ligands that are able to disrupt these inappropriately hyperstimulated pathways, by blocking SH2 domain-dependent interactions, may be developed to therapeutic agents for cancer, autoimmune disease, and chronic inflammatory disease.[2–4]

Recent several studies about peptide inhibitors for SH2 domains revealed that SH2 domain including *lck* and *src* exhibited a marked preference for the sequence -pYEEIE-, and that short peptides bearing this sequence exhibited a reasonably high affinity for the SH2 domains.[3–5] Even though moderately high-affinity phosphopeptide-based inhibitors for SH2 domains have been reported, their utility as therapeutic agents was limited mainly by their low stability against tyrosine phosphatase and protease and by low cell permeability.

As an alternative way, screening of natural products to identify small chemical inhibitors must afford useful and conceptually straightforward starting points in the development of SH2 domain inhibitors for therapeutic agents. Previously, we reported that a small chemical compound isolated from *Prunella vulgaris* had considerable inhibitory activities on *lck* SH2-pYEEIE-interaction, T-cell antigen receptor (TCR)-induced interleukin (IL)-2 expression, and subsequent T-cell proliferation in vitro.[6,7] Interestingly, the small chemical SH2 domain inhibitor was proved to be (*R*)-rosmarinic acid, frequently found in herbal plants.[8,9] Even though (*R*)-rosmarinic acid that did not contain phosphotyrosine moiety as a small chemical compound had several advantages for the lead compound over the phosphopeptide-based inhibitors, there are still some obstacles for in vivo efficacy test and for structure–activity relationship study as follows. Rosmarinic acid was not facilely isolated from natural sources as g scale and a total chemical synthesis of the compound was not economic because of several tedious syntheses and the low yield.[10,11] Furthermore, rosmarinic acid, dihydroxyl–phenyl ester compound, was reported to be hydrolyzed readily in vivo because of labile ester bond.[12]

In an attempt to identify new lead compound which have more improved in vivo stability and cell penetration

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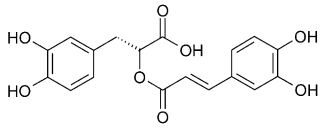
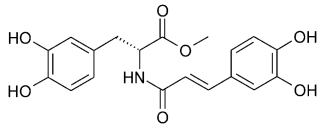
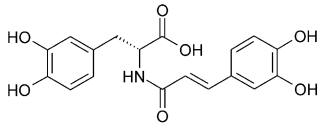
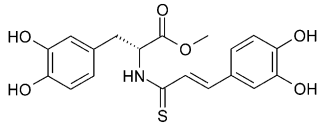
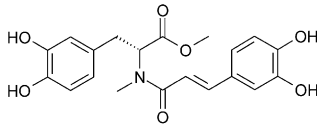
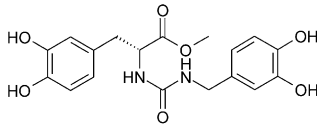
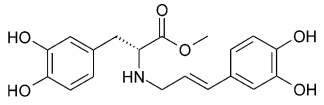
Table 1. The binding affinity of compounds for *lck* SH2 domain

Compd No.	Structure	IC_{50} (μM)
Rosmarinic acid		24
1		121
1a		20
2		162
3		149
4		98
5		> 500

Average IC_{50} values were calculated from three independent experiments performed in duplicate, which provided a standard deviation below 20%. IC_{50} value of control peptide (Ac-pYEEIE) was 1.5 μM.

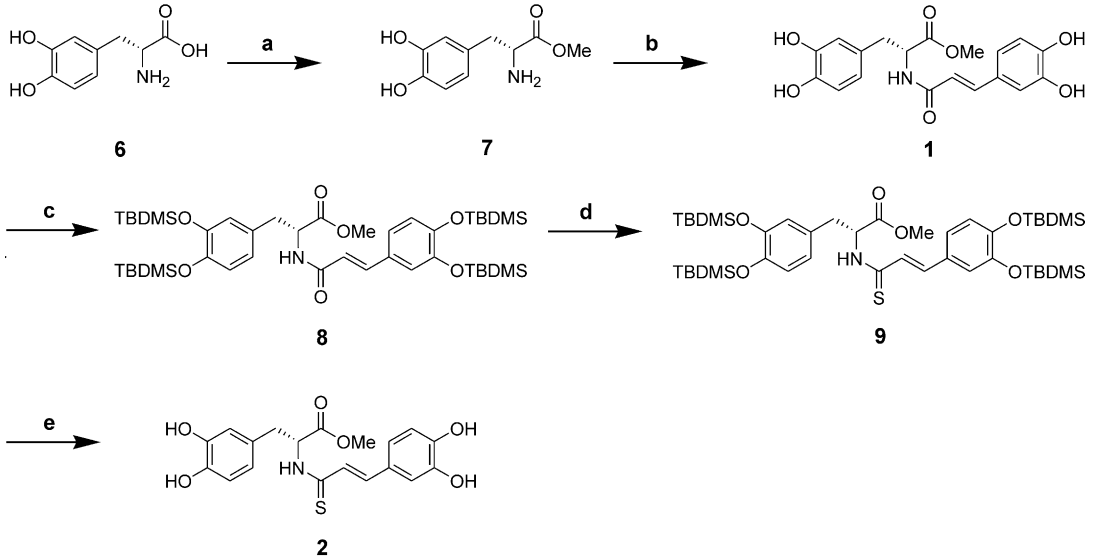
activity and can be easily synthesized, we designed and synthesized rosmarinic acid analogues, which had a different scaffold, and successfully identified novel lead compounds as inhibitors for *lck* SH2 domain.

Chemistry

In order to explore novel lead compound with more improved bioavailability, we designed the analogues of rosmarinic acid in which ester bond was replaced with amide bond, thioamide bond, *N*-methyl amide bond, reduced amide bond, and urethane bond (Table 1). In addition, to improve cell penetration, we prepared the analogues that had a methylester structure instead of an acid structure.

The syntheses of compounds **1** and **2** are shown in Scheme 1. Compound **1** was synthesized by coupling reaction of commercially available caffeic acid with 3,4-dihydroxylphenyl-D-alanine (DOPA) methylester **7**. Alternatively, compound **1** was prepared by two steps including the coupling reaction of 3,4-dimethoxycinnamic acid with DOPA methylester **7** and deprotection with BBr_3 (data not shown). However, both synthetic pathways gave the similar total yield (70%) regardless of the number of synthesis step. The protection of four hydroxyl groups of compound **1** and the reaction of compound **8** with Lawesson reagent,[13] followed by deprotection, provided compound **2** in 25% yield.

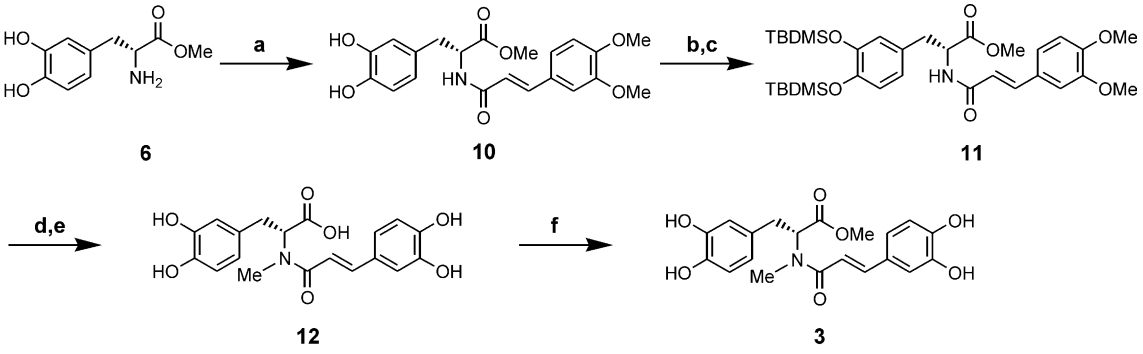
The synthesis of compound **3** was described in Scheme 2. The coupling reaction between DOPA methylester and 3,4-dimethoxycinnamic acid gave compound **10** in 87% yield. Protection of compound **10** and *N*-methylation, followed by deprotection with BBr_3 yielded compound **12** in a reasonable yield. However, treatment with NaH, followed by quenching in 1 M HCl



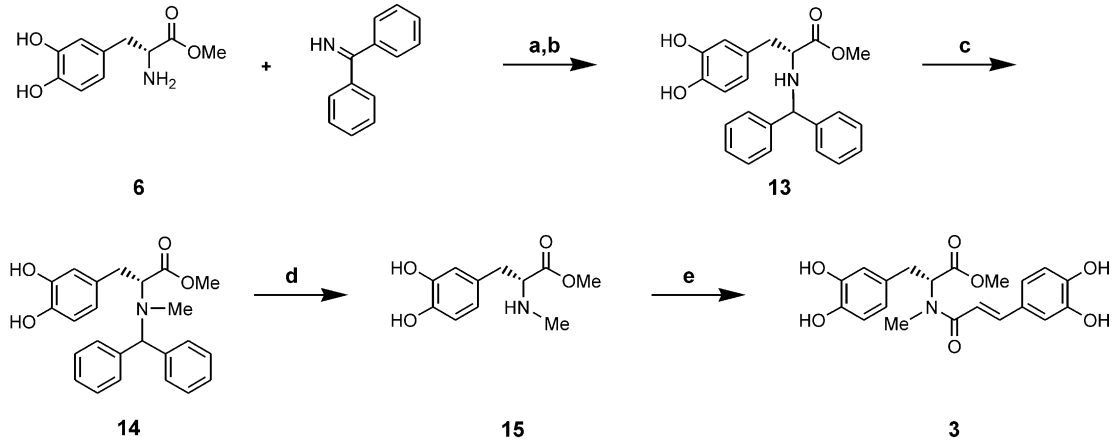
Scheme 1. Syntheses of compounds **1** and **2**. Reagent: (a) MeOH, $SOCl_2$, 0 °C; (b) caffeic acid, PyBOP, TEA, DMF, DCM; (c) TBDMSOTf, TEA, DCM; (d) Lawesson reagent, THF, reflux; (e) TBAF, THF.

hydrolyzed methylester bond of the compound. Thus, objective compound **3** was obtained by reacting compound **12** with methanol using $SOCl_2$ as activating agent. To consider the application of the synthesis scheme in solid-phase synthesis, we alternatively synthesized compound **3**, as shown in Scheme 3. In this case, we first synthesized *N*-methyl-3,4-dihydroxylphenyl-D-alanine (*N*-methyl DOPA) by reductive alkylation.[14] And then compound **3** was prepared by coupling *N*-methyl DOPA with caffeic acid. We obtained the best yield by using PyBroP among the several coupling agents such as DCC, DIPC, PyBOP, HBTU, and BOP, however the coupling yield (<20%) was still too low presumably because of steric hindrance of *N*-methyl group.

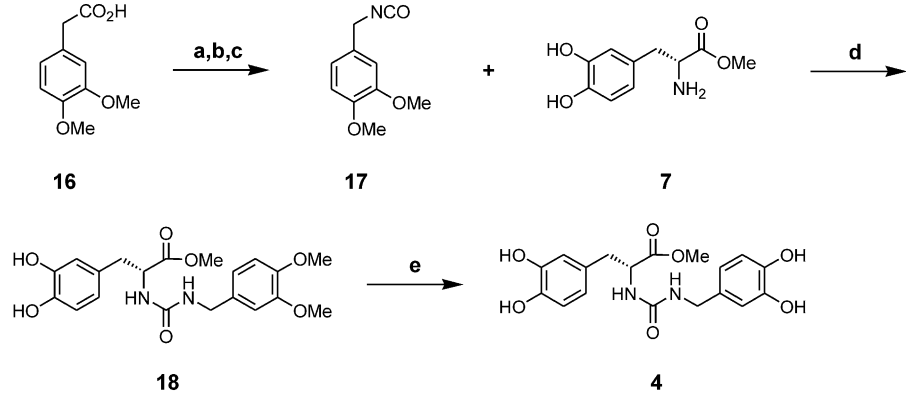
Compound **4** was synthesized as shown in Scheme 4. Compound **16** was converted into the corresponding isocyanate by Curtius rearrangement.[15] The reaction of the corresponding isocyanate with DOPA methylester, followed by hydrolysis of dimethylether of **18** provided compound **4** in a reasonable yield. As shown in Scheme 5, compound **5** was synthesized in two steps; reductive alkylation of DOPA methylester with the aldehyde and



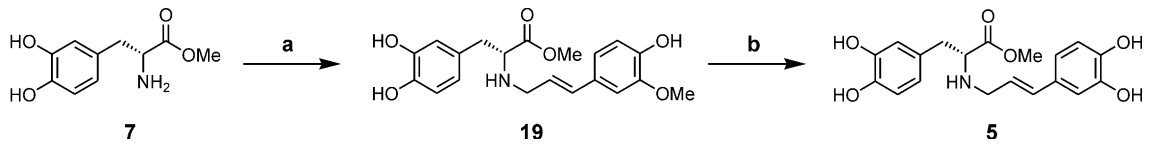
Scheme 2. Synthesis of compound **3**. Reagent: (a) 3,4-dimethoxycinnamic acid, PyBOP, TEA, DCM; (b) TBDMSOTf, TEA, DCM; (c) NaH, CH_3I, THF, quenching 1 M HCl (0 °C); (d) TBAF, THF; (e) 1.0 M BBr_3 in DCM, −30 °C → rt, DCM; (f) $SOCl_2$, MeOH.



Scheme 3. Synthesis of compound **3**. Reagent: (a) CH_2Cl_2, 24 h; (b) $NaCNBH_3$, AcOH, THF, 50 min; (c) $NaCNBH_3$, AcOH, HCOH, 6 h; (d) H_2, Pd/C, MeOH, 6 h; (e) PyBroP, 3,4-dihydroxycinammic acid, TEA, DMAP, DMF, 6 h.



Scheme 4. Synthesis of compound **4**. Reagent: (a) $SOCl_2$, 0 °C, DMF 2–3 drops, 5 h; (b) NaN_3, 0 °C, acetone/H_2O, 4 h; (c) benzene reflux, 5 h; (d) DCM, DMF, TEA, rt for 13 h; (e) BBr_3 in DCM 1.0 M solution, DCM, rt for 18 h.



7 19 5

Scheme 5. Synthesis of compound **5**. Reagent: (a) 4-hydroxy-3-methoxycinnamaldehyde, $NaCNBH_3$, AcOH (1%), THF, 4 h; (b) BBr_3 in DCM 1.0 M solution, −30 °C → rt, DCM, 5 h.

then deprotection of methyl ether bond of the phenyl ring. All synthesized analogues employed in this study were further purified by preparative reverse-phase HPLC and the products were obtained in high purity (generally >95% by RP analytical HPLC, $UV_{214\ nm}$) before binding assay for *lck* SH2 domain.

Results and Discussion

The inhibition activity of the derivatives of rosmarinic acid on *lck* SH2-AcpYEEIE- interaction was investigated by using the previously reported ELISA method.[16] All synthetic analogues except compound **5** inhibited the binding of EPQpYEEIPIYL with *lck* SH2 domain in a concentration-dependent manner (data not shown). Table 1 summarized IC_{50} values of compounds **1**–**5** obtained in the ELISA assay.

As shown in Table 1, we successfully identified novel small chemical inhibitors with a different scaffold from that of rosmarinic acid. However, most of the analogues exhibited lower binding affinity than rosmarinic acid. As all analogues had the same structure as the lead compound except the backbone scaffold and methylester substructure, backbone scaffold or acid substructure must play an important role in the binding activity. To investigate which modification is more dominant for the activity, compound **1a** that had acid substructure was prepared by hydrolysis of compound **1** and its binding affinity with *lck* SH2 domain was measured. Compound **1a** had a similar binding affinity to the lead compound, rosmarinic acid, which indicated that the decrease of binding affinity of the analogues was mainly due to the modification of acid substructure. As shown in Table 1, the comparison between IC_{50} value of compounds **1**–**5** indicated that the binding activity must rely on the rigidity of the scaffold between two catechol substructures because inactive analogue, compound **5**, had the most flexible scaffold among the analogue series. Compound **4** showed the best binding affinity among the series, which indicated the double bond of the compounds is not required for the full activity and if some chemicals had conformational constrain between two catechol substructures, the compound must exhibited considerable binding affinity with *lck* SH2 domain.

We expect that compound **1** containing amide bond must have different bioavailability from that of rosmarinic acid. However, it is difficult to anticipate that compound **1** may exhibit improved bioavailability because amide bond did not have a considerable resistance against peptidase but esterase, whereas rosmarinic acid had a resistance against peptidase rather than esterase. Compound **2** containing thioamide bond must have improved bioavailability because thioamide bond was reported to be more stable than amide bond in vivo.[18] However, compound **2** showed the lowest inhibition activity among the active analogue series, which can be explained by the fact that the larger and less electronegative sulfur atom than oxygen atom must induce some conformational distortions and/or may form hydrogen bonding to the protein less tightly.

Considering the inhibition activity as well as stability and cell penetration activity, compound **3** must be better candidate for in vivo efficacy test than the compounds **1**, **2**, and **4** because *N*-methyl amide bond had a potent resistance against various intracellular enzymes and are more hydrophobic than amide bond and urethane bond.

In the present study, we successfully identified novel lead inhibitors with a different scaffold from that of rosmarnic acid for *lck* SH2 domain and characterized the role of backbone and acid substructure of the inhibitors on the binding activity. On the basis of the structure of compound **1a** as a lead chemical, several non-phosphopeptide inhibitors for SH2 domains were successfully synthesized from solid phase parallel synthesis[17] and structure–activity relationships studies of (*R*)-rosmarinic acid and compound **1** are in progress.

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